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Review Article

An Evaluation of the Mode of Action Framework for Mutagenic Carcinogens Case Study: Cyclophosphamide

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In response to the 2005 revised US Environmental Protection Agency (EPA) Cancer Guidelines, a Risk Assessment Forum's Technical Panel has devised a strategy in which genetic toxicology data combined with other information are assessed to determine whether a carcinogen operates through a mutagenic mode of action (MOA). This information is necessary for EPA to decide whether age-dependent adjustment factors (ADAFs) should be applied to the cancer risk assessment. A decision tree has been developed as a part of this approach and outlines the critical steps for analyzing a compound for carcinogenicity through a mutagenic MOA (e.g., data analysis, determination of mutagenicity in animals and in humans). Agents, showing mutagenicity in animals and humans, proceed through the Agency's framework analysis for MOAs. Cyclophosphamide (CP), an antineoplastic agent, which is carcinogenic in animals and humans and mutagenic in vitro and in vivo, was selected as a case study to illustrate how the framework analysis would be applied to prove that a carcinogen operates through a mutagenic MOA. Consistent positive results have been seen for mutagenic activity in numerous in vitro assays, in animals (mice, rats, and hamsters) and in humans. Accordingly, CP was processed through the framework analysis and key steps leading to tumor formation were identified as follows: metabolism of the parent compound to alkylating metabolites, DNA damage followed by induction of multiple adverse genetic events, cell proliferation, and bladder tumors. Genetic changes in rats (sister chromatid exchanges at 0.62 mg/kg) can commence within 30 min and in cancer patients, chromosome aberrations at 35 mg/kg are seen by 1 hr, well within

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the timeframe and tumorigenic dose range for early events. Supporting evidence is also found for cell proliferation, indicating that mutagenicity, associated with cytotoxicity, leads to a proliferative response, which occurs early (48 hr) in the process of tumor induction. Overall, the weight of evidence evaluation supports CP acting through a mutagenic MOA. In addition, no data were found that an alternative MOA might be operative. Therefore, the cancer guidelines recommend a linear extrapolation for the risk assessment. Additionally, data exist showing that CP induces mutagenicity in fetal blood and in the peripheral blood of pediatric patients; thus, the ADAFs would be applied. Environ. Mol. Mutagen. 49:117–131, 2008. Published 2008 Wiley-Liss, Inc.†

Key words: mutagenicity; cyclophosphamide; mode of action; cytotoxicity

INTRODUCTION

Cyclophosphamide (CP) [*N*,*N*-bis(2-chloroethyl) tetrahydro-2H-1,3,2-oxphosphorin-2-amine, 2-oxide monohydrate], also known as Cytoxan or Endoxan, is a pharmaceutical product used as an antineoplastic agent in the treatment of a wide range of cancers including Hodgkin's disease, non-Hodgkin's lymphoma, many types of leukemia, multiple myeloma, neuroblastomas, adenocarcinomas of the ovary, and certain malignant neoplasms of the lung. It is also used as an immunosuppressant agent for arthritis, scleroderma, glomerulonephritis, chronic hepatitis, multiple sclerosis, and organ transplantation.

CP is generally administered to humans orally (100–200 mg/kg, daily) or intravenously (600–1,000 mg/m^2 every 3–4 weeks) for cancer treatment. Although CP is an effective drug in the treatment of a host of diseases, IARC [1981] designated CP as carcinogenic to humans based on data showing an association between treatment for nonurothelial primary cancers, rheumatoid arthritis, and lupus and an increased incidence of secondary bladder neoplasms. CP treatment is also linked to leukemia in patients treated for other primary cancers and non-neoplastic diseases.

In animals, malignant tumors were observed in male and female Sprague Dawley rats orally administered CP in drinking water in a lifetime study. Tumors included the following: transitional-cell carcinomas of the urinary bladder; an increased risk of developing tumors of the hematopoietic tissue; and neurological sarcomas arising from peripheral nerves [IARC, 1981]. In other rat studies using either intraperitoneal (i.p.) or intravenous (i.v.) administration, CP induced malignant tumors including the following: bladder carcinomas, reticulum-cell sarcomas, hemangioendotheliomas in various organs, osteosarcomas, and neoplasms of the lung, liver, testis, and mammary gland. CP produced multiple neoplasms (mammary carcinomas, pulmonary sarcomas and adenomas, and liver tumors) when subcutaneous, intramuscular, or i.p. injections were selected as the exposure routes for mice. Following pre- and postnatal exposures, pregnant mice, receiving CP by i.p. injection showed hepatomas, lung carcinomas, and skin carcinomas in mice after 60 weeks. Male and female offspring developed lung adenomas and carcinomas; females also had hepatomas and skin carcinomas and sarcomas. Additionally, CP increases the incidence of developmental anomalies in the offspring of mice and rats [Anderson et al., 1995]. It is also a well-established in vitro and in vivo mutagen [Anderson et al., 1995; Waters et al., 1988; and [Lohman and Lohman, 2000].

The purpose of this data analysis is to use CP as a case study to illustrate how the EPA would undertake a mode of action (MOA) analysis for a carcinogen with mutagenic activity. This analysis is in accordance with the EPA's 2005 Guidelines for Carcinogen Risk Assessment [USEPA,

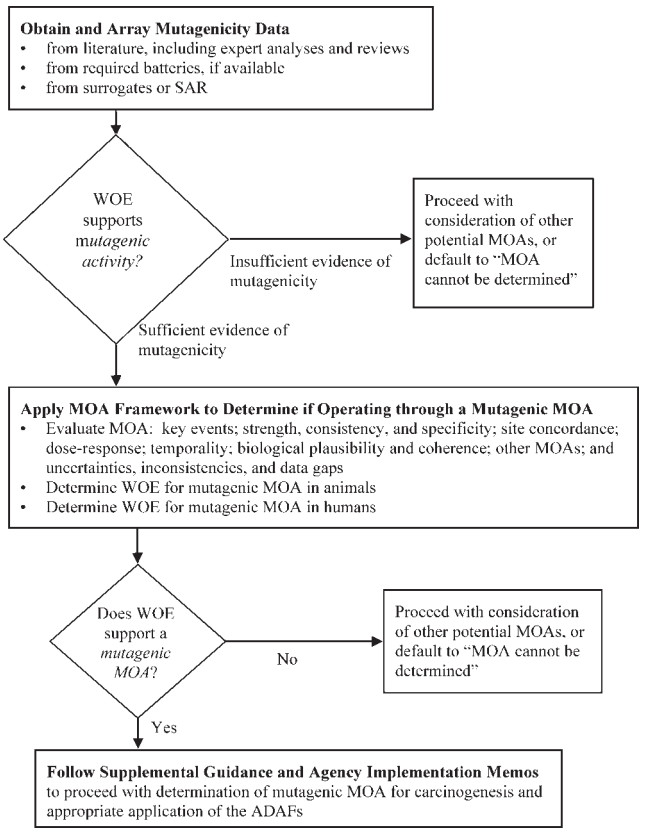

Fig. 1. Mutagenic MOA decision tree [Adapted from Dearfield and Moore, 2005].

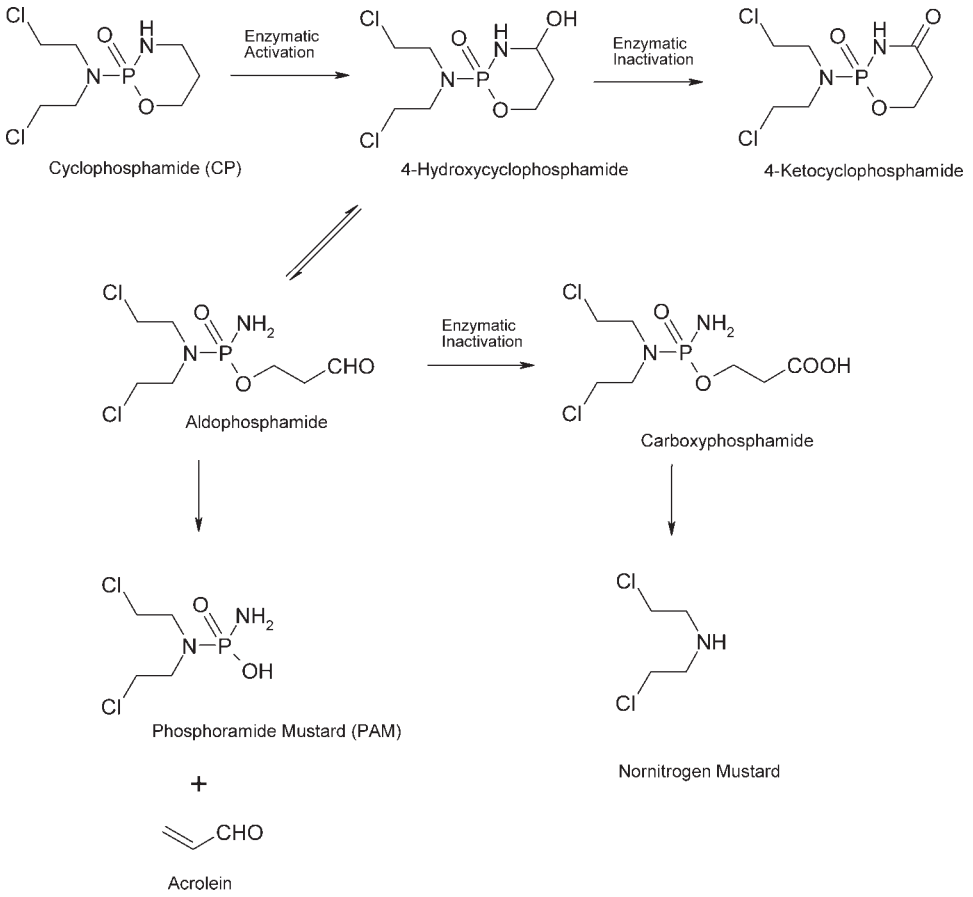

Fig. 2. Metabolic pathway of cyclophosphamide.

2005a] and the Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens, particularly, agents with a mutagenic MOA [USEPA, 2005b].

MATERIALS AND METHODS

Genotoxicity Data Collection

A framework for the analysis of carcinogens with a mutagenic MOA has been developed which utilizes a decision tree as part of the general approach for analysis of a mutagenic MOA (Fig. 1). As outlined in the decision tree, the first step in this process is to gather and organize genotoxicity data and determine if the criteria established to provide sufficient evidence of mutagenicity could be satisfied. The second step is to determine whether a mutagenic MOA for carcinogenesis can be demonstrated in animals and if, this mutagenic MOA is supported in humans. For this effort, data were extracted from the relevant literature on mutagenicity, toxicity, and carcinogenicity to decide if a link can be made between cyclophosphamide-induced tumors and mutagenicity. Our basic approach has been presented in an earlier publication [McCarroll et al., 2002].

Metabolism

The chemical structure for CP is illustrated in Figure 2. As shown, CP is initially oxidized to carboxyphosphamide, which leads to the formation of a potent alkylating agent, nornitrogen mustard. Another pathway yields acrolein and phosphoramide mustard (PAM). PAM is the major cytotoxic agent produced from the metabolism of CP and listed by Anderson et al. [1995] as the metabolite largely responsible for the antineoplastic response and alkylation of DNA (Boyd et al., 1986 as cited in Anderson et al., 1995]). Acrolein, a highly toxic metabolite, is thought to be involved in the toxic side effects of CP.

Genetic Toxicology Data

In vitro and in vivo genetic toxicology data from the open literature were surveyed as previously described [Dearfield et al., 1993], and the genetic activity profile (GAP) for CP was developed jointly with the International Agency for Research on Cancer (IARC) to graphically display genetic toxicology data as a function of concentration or dose. Details for the schematic representation of the GAP for CP, depicted in Figure 3, can be found in Waters et al. [1988] and in Lohman and Lohman [2000]. The GAP is a reliable starting point for gathering mutagenicity data but should be followed by an extensive literature search. Data from the cited literature were briefly reviewed for general quality. No major study deviations from regulatory guidelines were seen in the cited literature used in this undertaking.

Carcinogenicity/Mode of Action Data

Carcinogenicity data were obtained from mouse and rat studies reviewed in IARC Monographs [IARC, 1981]. In addition, case reports and epidemiological studies of carcinogenicity, presented by IARC, were summarized. Relevant data pertaining to various phases of the MOA

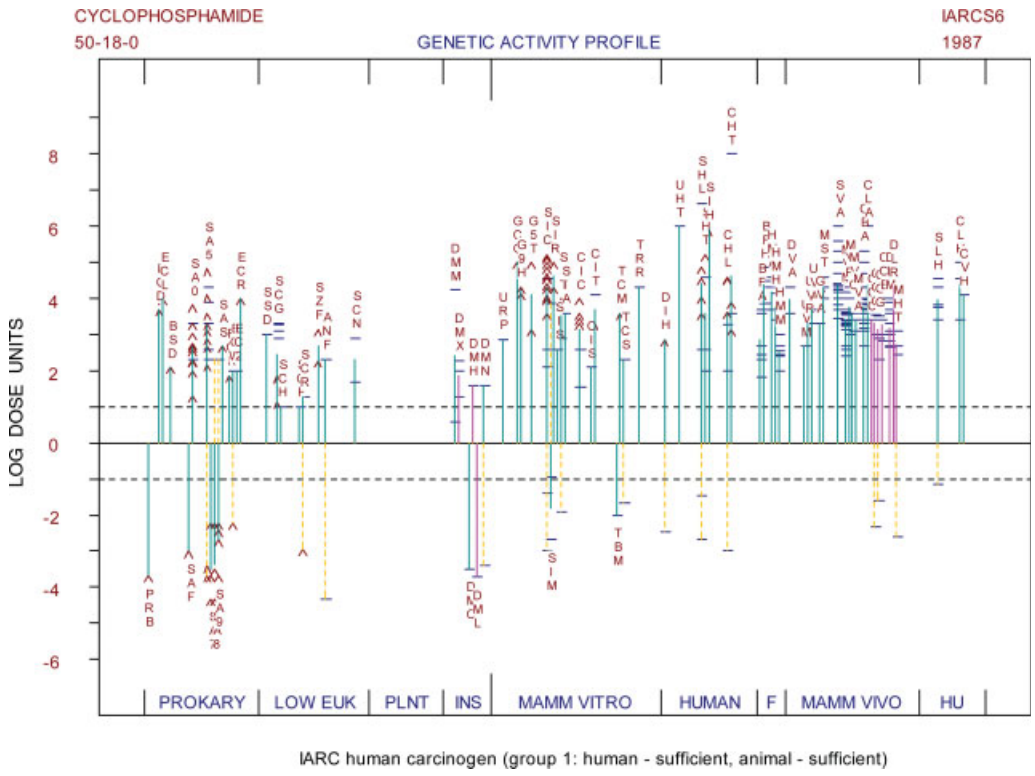

Fig. 3. Cyclophosphamide genetic toxicology profile [Adapted from Waters et al., 1988].

analysis (i.e., description of the postulated MOA, key events, dose response, temporal associations, and biological plausibility, other MOAs, and relevance to humans) were derived from studies reviewed by IARC and from the open literature.

CRITICAL ANALYSIS OF THE AVAILABLE DATA

Genetic Toxicology

In Vitro Test Systems

There is an extensive database on mutagenicity for CP compared with most mutagenic agents encountered in environmental risk assessments. Based on the genetic activity profile (GAP) developed by Waters et al. [1988] for CP (Fig. 3), induction of gene mutations has been reported only after metabolic activation in bacteria (*Salmonella typhimurium*, *Escherichia coli*), yeast (*Saccharomyces cerevisiae*), and mammalian cells (mouse lymphoma L5178Y). In *Drosophila melanogaster*, it induces somatic mutations, recessive lethal mutations, and chromosome aberrations in somatic and/or germinal cells. S9-activated CP is also clastogenic in vitro in Chinese hamster ovary cells (CHO), Syrian hamster cells, and human lymphocytes. Sister chromatid exchange (SCE) induction has been reported in CHO, V79, Syrian hamster and a variety of other mammalian cell lines when tested in the presence of S9 activation. Concentrations up to 12 μg/ml with S9 increased the frequency of micronuclei in V79 cells and human lymphocytes. Apart from SCE, there is also clear evidence of other types of DNA damage (DNA repair in bacteria, unscheduled DNA synthesis in primary rat hepatocytes, and *Hela* cells and DNA adducts in calf thymus DNA). In virtually every positive in vitro genetic toxicology assay, positive results are achieved in the presence of S9 activation at concentrations well below limit and/or cyctotoxic levels. The lowest effective concentration (i.e., the lowest concentration or dose showing a positive effect) found in an in vitro assay was S9-activated 0.024 μg/ml CP in the SCE assay performed in human peripheral lymphocyte [Clare et al., 1982]. These findings are consistent with the fact that mutagenic agents are usually positive in vitro for multiple genetic toxicology endpoints and are active in an increasingly higher order of phylogenetically distinct species.

In Vivo Test Systems (Whole Animals)

Gene mutation assays. Until recently, the only in vivo gene mutation assay available for CP was the somatic cell mouse spot test. Findings reported by Anderson et al. [1995] from two studies indicate that CP, administered subcutaneously at doses of 5–45 mg/kg to females on day 10 of pregnancy, was mutagenic, causing a dose-related increase in the number of offspring with mosaic

TABLE I. Summarized Results from Positive In Vivo Cytogenetic Assays with Cyclophosphamide in Mammals

Endpoint	Dose/route (no. of doses)	Time of evaluation	Cell type	Reference[a]
Mouse				
Sister chromatid exchange	0.5–40 mg/kg/i.p. (1)	3–40 hr	Bone marrow	Cole et al., 1983
Chromosome aberrations	5–500 mg/kg/i.p. (1)	6–96 hr	Bone marrow	Salvadori et al., 1992
	1–40 mg/kg/i.p. (1)	24 hr	Bone marrow	Rossi et al., 1982
	5–15 mg/kg (1)	6–24 hr	Embryonic cells	Braun et al., 1986
	13.3–119.7 mg/kg/i.p. (2–5)	8 hr after last dose	Bone marrow	Rohrborn and Basler, 1977
	140–730 mg/kg/ (continuous) drinking H_2O	4–28 days	Bone marrow	Sram et al., 1981
	50 mg/kg/i.p. (2)	6 hr after last dose	Bone marrow	Gad-El-Karim et al., 1984
Micronuclei	5–100 mg/kg/i.p. (1)	10–72 hr	Fetal blood	King and Wild, 1979
			Bone marrow	MacGregor et al., 1980
			Peripheral blood	Hutter and Strohr, 1982
			Colon epithelium	Salamone et al., 1980, 1982
	10–500 mg/kg/i.p. (2)	6 hr after last dose	Bone marrow	Salamone et al., 1980; Ghaskadbi et al., 1992
	22 mg/kg/i.p. (2)	72 hr after last dose	Blood	Schlegel and MacGregor, 1983
Rat				
Sister chromatid exchange	5–50 mg/kg/i.p. (1)	0.5–6 hr	Lymphocytes	Dearfield et al., 1985
	0.62–50 mg/kg/i.p. (1)	30–72 hr	Bone marrow Splenocytes	Simula and Priestly, 1992
Chromosome aberrations	20–75 mg/kg/i.p. (1)	6–24 hr	Bone marrow	Krishna et al., 1991
	10–40 mg/kg/i.p. (1)	22–24 hr	Bone marrow	
	5–45 mg/kg/i.p. (2)	8 hr after last dose	Bone marrow	Rohrborn and Basler, 1977
	20–100 mg/kg/i.p. (5 days)	End of treatment	Bone marrow	Neda et al., 1977
	36–230 mg/kg (continuous) drinking H_2O	End of treatment	Bone marrow	Topinkova and Sram, 1982
Micronuclei	10–40 mg/kg/i.p. (1)	24 hr	Bone marrow	Barbarasa et al., 1979
	10–50 mg/kg/i.p. (2)	24 hr after last dose	Bone marrow	
Hamster				
Chromosome aberration	35–140 mg/kg/i.p. (1)	6–72 hr	Bone marrow	Shev et al., 1990
	13.3–120 mg/kg/i.p. (2)	8–24 hr after last dose	Bone marrow	Rohrborn and Basler, 1977
	2–40 mg/kg/i.p. (2–5)	6–24 after last dose	Bone marrow	Rohrborn and Basler, 1976
Micronuclei	13–120 mg/kg/i.p. (2)	2–24 hr after last dose	Bone marrow	Rohrborn and Basler, 1979

[a]As cited in Anderson et al. [1995].

coats and/or white ventral spots. The maximum mutagenic response was seen at 10 mg/kg; doses $\geq$15 mg/kg were lethal. In the second study, similar positive results were achieved at 2.5 mg/kg and severe toxicity to the pups occurred at 10 mg/kg following i.p. injection on days 8–10 of pregnancy.

Cyclophosphamide has been used by Myhr [1991] to validate the Muta™ Mouse assay with the *lacZ* gene as the indicator of mutation. In this study, CP administered i.p. at 100 mg/kg for 5 days to male transgenic mice was positive in bone marrow harvested 7 days after the last treatment, producing a threefold increase in the mutation frequency (MF). In a more elaborate study of tissue specificity, Gorelick et al. [1999] applied a single i.p. injection of 100 mg/kg to adult male B6C3F1 *lacI* transgenic mice; necropsies were performed 6 weeks posttreatment and selected tissues were harvested. Results showed significant increases in the MF in the target sites for tumorigenesis (lung and urinary bladder) but not in the kidney, bone marrow or splenic T-lymphocytes. The spectra of mutations observed by Gorelick et al. [1999] from the lung and the urinary bladder indicated a significant increase in the frequency of A· T→ T· A transversions and an elevated frequency of deletions. The lack of a mutagenic response in bone marrow or splenic T-lymphocytes conflicts with the positive data (bone marrow) in the Myhr study [1991] and the increased MFs for splenic lymphocytes shown by Walker et al. [1999] at the *Hprt* gene but not the *lacI* transgene in male transgenic B6C3F1mice. In the latter study, both genes were examined for CP-induced mutagenicity in transgenic mice receiving single i.p. injections of 0, 25, or 100 mg/kg CP and sacrificed 6 week posttreatment. The lack of an effect at the lacI transgene led Gorelick et al. [1999] to conclude that "the *lacI* system is less sensitive that the *Hprt* gene, at least in splenic cells." Lambert et al. [2005] in their detailed review of transgenic rodent mutation assays list generally positive results for CP bone marrow harvested from animals in the MutaMouse test system 3–7 days postexposure to five daily doses of 500 mg/kg CP. Thus the target site concordance between positive bone marrow and splenic cells and leukemias and lymphomas in animals and human has been established. Although there are conflicts in the in vivo mutational assays, issues

TABLE II. Summarized Positive Results from In Vivo Genetic Toxicology Assays with Cyclophosphamide in Humans

Endpoint	Exposure type[a]	Dose/time between dosing (no. of doses)	Exposure duration or sample time	Cell type	Reference
Sister chromatid exchange	Chemotherapy patients	5–14 mg/kg/24 h (180–240)	6–8 months	Lymphocytes	Schuler et al., 1979[b]
	Chemotherapy patients	35 mg/kg (1)	1–5 hr	Lymphocytes	Musilova et al., 1979[b]
	Chemotherapy patients	18–24 mg/kg (1–5)	1–7 days	Lymphocytes	Duker, 1981[b]
Chromosome aberrations	Chemotherapy patients (Children—Nonmalignant conditions)	3–5 mg/24 hr (180–240)	1–2, 3–4, 6 months (during treatment)	Lymphocytes	Dobos et al., 1974[b]
			1, 6–15 months posttreatment		
	Chemotherapy patients (unspecified malignant tumors or acute glomerulonephritis	2,300–3,000 mg/(daily)	3 week/2–24 week	Lymphocytes	Musilova et al., 1979[b]
	Chemotherapy patients	1–7 mg/kg (180–240)/ 24 hr (180–240)	6–8 months	Lymphocytes	Schuler et al., 1979[b]
	Chemotherapy patients (recurrent ovarian cancer)	2.0 g (1)	3 or 24 hr posttreatment	Lymphocytes	Morad and El Zawahri, 1977[b]
	Chemotherapy patients	40 mg/kg (1)	24 hr posttreatment	Bone marrow	Goetz et al., 1975
Micronuclei	Chemotherapy patients	40 mg/kg (1)	24 hr posttreatment	Bone marrow	Goetz et al., 1975
DNA damage (comet assay)	Chemotherapy patients (breast cancer)	600–1,800 mg/m^2 (1)	Pretreatment and 1–21 hr post	Lymphocytes	Vaghef et al., 1997
Gene mutations *Hprt* locus	Chemotherapy patients	750 mg/m^2 /1 month (6)	6 months/14–28 days	Lymphocytes	Ammenhuser et al., 1988[b]

[a]Administration route for all studies was intravenous.
[b]As cited in Anderson et al. [1995].

related to test system specificity were explained. Based on the wealth of positive animal data and using a weight of the evidence approach, it was concluded that CP was detected as an in vivo mutagen in mouse cancer target tissues. No transgenic rat studies were found in the open literature.

Cytogenetic assays. As shown in Table I, data extracted from tables prepared by Anderson et al. [1995] indicate that CP, administered generally by i.p. injection, induced significant and/or dose-related increases in SCE, chromosome aberrations, and/or micronuclei primarily in bone marrow harvested from mice, rats, or hamsters following single or multiple exposures. Of note was a time course study conducted by Dearfield et al. [1985] in the bone marrow of Fischer 344 rats treated once with 5, 10, or 20 mg/kg CP (i.p.). The authors stated that a gradual dose-related increase in SCE induction started as early as 30 min after exposure. There is also evidence that CP administered once by i.p. injection (20, 50, or 100 mg/kg) to female mice on the 14th, 15th, and 16th day of pregnancy, induced higher frequencies of micronucleated polychromatic erythrocytes (MPCEs) in fetal blood than in maternal bone marrow. At 20 or 100 mg/kg, the increases in MPCEs were 66- or 75-fold higher in fetal blood compared with 15- or 43-fold increases in maternal bone marrow, respectively [King and Wild, 1979]. The lowest effective dose was reported by Simula and Priestly [1992] to be 0.62 mg/kg for SCEs in rat bone marrow; a higher dose (1.25 mg/kg) was required for mice. For both species, the effect was significant ($P < 0.05$) and dose-related.

Genetic toxicology studies in humans. Since CP has a long history of clinical use, Anderson et al. [1995] cite two sources of human exposure data for CP: hospital settings workers (nurses, pharmacists, doctors, and patients) and workers in the manufacturing setting. For example, Burgaz et al. [1999] reported significant increases in micronuclei in peripheral blood lymphocytes and buccal epithelial samples collected from all nurses ($n = 26$) handling CP. It should be noted that controls were matched for age, sex, and smoking habits and that CP excretion rates for exposed nurses ranged from 0.2 to 9.14 μg/24 hr. Data from other studies compiled by Anderson et al. [1995] or present in the open literature on patients receiving CP are summarized in Table II. As shown, studies examining structural aberrations, SCEs, gene mutations, or DNA damage (e.g., Comet assay) were positive in lymphocytes harvested either from the peripheral blood or the bone marrow of cancer patients or individuals treated for other conditions. There is even a positive chromosome aberration study in the lymphocytes of children being treated for nonmalignant conditions [Dobos et al., 1974]. In this study, 26 children (17 M 9 F, ages 2–17) were treated with 3–5 mg/kg CP for 6–8 months. Analysis of peripheral lymphocyte samples 4–6 weeks after treatment commenced revealed a significant, 18.6% increase in

chromosome breakage. Significance ($P < 0.01$) persisted with a 15% increase still recorded 4–6 weeks beyond termination of therapy and a returned to control levels 6–7 months after termination of therapy. For gene mutations, Khan et al. [1998] examined bladder tumors from resected or biopsy tissues of 18 patients diagnosed with primary bladder cancer. They ranged in age from 38 to 67 years and were composed of 14 males and 4 females who received cumulative doses of 6–125.2 mg/kg CP. Using polymerase chain reaction (PCR) amplification and DNA sequencing, the authors found that 43% (9/19) of the tumors had a mutation in the *p53* tumor suppressor gene as compared with a 6% frequency for sporatic bladder cancer. The predominant mutation reported by Kahn et al. [1998] was at G: C bp (7/9, 78%), a preference for non-Cp-G sites (86%), and 71% with G:C →A:T transitions. The investigators stated that this mutation spectrum for humans matches the phosphoramide mustard adduction sequences determined in a repetitive primer-extension assay indicating that this metabolite might be the key mutagen in cyclophosphamide-related bladder cancer. However, these data conflict with the mutational spectra reported by Gorelick et al. [1999] for the lung and the urinary bladder of CP-treated transgenic mice which indicated a significant increase in the frequency of A· T→ T· A transversions. This is not unexpected since Gorelick et al. [1999] noted that the available DNA adduct data and cytogenetic data suggest that CP treatment leads to several classes of mutations including base substitutions, frameshifts, and deletions. Similarly, the in vivo transgenic gene mutation assays were designed to inform site concordance between target tissue mutagenicity and tumor induction. For this reason, sensitive exogenous gene systems were constructed to serve as surrogates not as definitive evidence of the punitive mutational spectra for cancer induction.

In another study that detected somatic cell mutations, 6-thioguanine-resistant (TG^r) T-lymphocytes, from multiple sclerosis patients treated for 4-week intervals with i.v. infusions of 750 mg/m^2, were significantly ($P < 0.05$) increased 2 weeks after the first dose [Ammenheuser et al., 1988].

These positive findings for gene mutations and chromosomal aberrations in different animal and human tissues are consistent with the observed pattern that agents with a potential mutagenic MOA generally demonstrate (i.e., positive responses in at least one in vivo genetic toxicology assay).

DNA Adducts

A key consideration for a mutagenic carcinogen in the carcinogenesis process is DNA damage manifested as DNA adducts [Butterworth, 2006]. Accordingly, the following information was gathered on DNA adduct formation.

Whole animals. Several investigators have reported DNA adduct formation in mice and rats exposed to CP. In an initial study, Hemminki [1985] used a rat liver microsomal system to study the binding of [chloroethyl-3H]cyclophosphamide to DNA and found that 100 μCi CP binds to mouse DNA. In the subsequent in vivo studies, mice were given injections i.p. of 100 μCi of cyclophosphamide and adducts were formed in mouse liver, kidney, lungs, and the bladder within 1–7 hr after treatment, with the greatest increase in adducts in the mouse lung. The major adduct was identified by Benson et al. [1988] as *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl) ethyl] amine and was detected in rats injected i.p. with 2.775 MBq [^{3}H]cyclophosphamide. The maximal levels of binding to DNA occurred between 1 and 4 hr with the highest binding levels observed in the rat bladder. In another study, Maccubbin et al. (1991, as cited by [Anderson et al., 1995]) found DNA adducts in the liver of mice injected i.p. with 250 mg/kg CP and sacrificed 8 hr after treatment. Povirk and Shuker [1994], in their review of DNA damage and nitrogen mustards state that the major alkylating product of CP appears to be phosphoramide mustard (PAM, see Fig. 2) which gives rise to a series of mono- and crosslinked adducts of nornitrogen mustard: guanine-N7 (nor-G), guanine-N7 (nor—G—OH), and phosphotriester. Noting the strong structure activity relationship for nitrogen mustards, the authors further state that "all nitrogen mustards induce monofunctional guanine-N7 adducts, as well as interstrand N7-N7 crosslinks involving the two guanines in GNC · GNC ($5'\rightarrow3'$/$5'\rightarrow3'$) sequences." This statement agrees with the previously discussed findings for DNA adduction in animals.

Humans. No data were found in the open literature on DNA adducts in humans. Nevertheless, the presented data are robust and support the conclusion that CP is DNA-reactive in humans.

In summary, based on these findings of DNA reactivity and mutagenicity for both animals and human, it was concluded that there is sufficient evidence from a wide range of in vitro and in vivo genetic toxicology assays to indicate that CP is mutagenic in animals and in humans.

Carcinogenicity Data

Animals

IARC [1981], citing the study of Schmähl and Habs [1979] states that CP is carcinogenic in rats after oral exposure of 40 male and 40 female Sprague Dawley rats through drinking water containing 0.31, 0.63, 1.25, or 2.5 mg/kg/day CP five times per week for life. It is noteworthy that the doses used in the lifetime study were below 1% of the LD_{50}. Combined malignant tumors showed significant increases in frequency for male and female rats at doses $\geq$0.63 mg/kg/day. However, the primary tumor of interest is urinary bladder cancer (mainly seen in male rats) because this tumor type also occurs in

TABLE III. Urinary Bladder Tumors in Male Sprague Dawley Rats Receiving Cyclophosphamide in Drinking Water Five Times/Day (Lifetime)

	Dose (mg/kg/day)				
	Males				
Tumor type	0	0.31	0.63	1.25	2.5
Urinary bladder[a]	0/38 (0%)	2/34 (6%)	2/36 (5%)	5/35* (14%)	7/31** (23%)

Extracted from Schmähl and Habs [1979].

[a]Other neoplasms included: neurogenic sarcomas arising from peripheral nerves and tumors of the hematopoietic system at ≥0.63 mg/kg.

*$P < 0.05$,

**$P < 0.01$: bias due to deaths was eliminated.

TABLE IV. Tumors in Male and Female New Zealand Black/New Zealand White Hybrid Mice Receiving Daily Subcutaneous Injections of Cyclophosphamide (Lifetime)

	Dose (mg/kg/day)		
	Males and females[b]		
Tumor type[a]	0	5.7	16
Total neoplasms	0/13 (0%)	15/15 (100%)	17/19 (90%)
Multiple neoplasms	0/13 (0%)	11/15 (73%)	8/19 (42%)

Extracted from IARC [1981].

[a]Tumors included: mammary carcinomas (16 at 16 mg/kg and 5 at 5.7 mg/kg) other tumors were lymphomas and pulmonary adenomas and sarcomas IARC [1981].

[b]Combined for both sexes; animals were observed for life or sacrificed when moribund or when tumors developed.

humans treated with CP. As presented in Table III, significant pairwise and dose-related increases in urinary bladder tumors were seen at the two highest doses (1.25 and 2.5 mg/kg/day). Table IV presents data showing that CP is also carcinogenic in New Zealand Black/White (NZB/NZW) mice when administered by subcutaneous (s.c.) injection to two groups (17 M; 21 F), producing total neoplasms in 17/19 mice at 16 mg/kg/day and 15/15 at 5.7 mg/kg/day. Malignant tumors were classified primarily as mammary carcinomas (16 at 16 mg/kg/day, five at 5.7 mg/kg/day) but pulmonary adenomas and sarcomas were also observed. None of the control animals had tumors. Early deaths in the high dose group were associated with tumor formation (time of deaths were not provided). In a second study using NZB/NZW hybrid mice, groups of 10 males and 10 females received s.c. injections of 1 or 8 mg/kg/day for 93 weeks. Eight males and nine females of the high dose group developed tumors (primarily generalized lymphoreticular and poorly differentiated sarcomas; squamous cell carcinomas were also seen at the injection site in three females). Animals administered the low dose had tumor incidences comparable to the control group. Tumors were observed after 60 weeks of treatment in males and 40 weeks in females.

The above studies were selected as representative to illustrate the carcinogenicity of CP in rodents. There are, however, many other studies listed by IARC [1981] in either rats or mice, which support the evidence that CP is carcinogenic in both sexes of both rodent species using various routes of exposure. Based on the type of tumors induced in rats (mainly urinary bladder carcinomas, tumors of the hematopoietic system, and neurogenic sarcomas) and mice (largely mammary carcinomas, lymphomas and pulmonary adenomas and sarcomas), it was concluded that CP is a systemic carcinogen (i.e., an agent that causes tumors at sites that are distal from the portal of entry, regardless of the route of exposure). This conclusion is in accord with the assessed mutagenicity data showing that CP is a systemic mutagen.

Humans

IARC [1998] states that there is epidemiological evidence that CP increases the incidence of bladder cancer, and there is a suggestion that the incidence of other cancers may also be increased. Case reports of bladder cancer and acute nonlymphocytic leukemia linked to CP therapy also exist. Reports include the observation of excess frequencies of bladder cancer following treatment of patients with CP for nonmalignant diseases such as rheumatoid arthritis (Kinlen et al., 1981 as cited in IARC, 1998]). In the comprehensive study of Pedersen-Bjergarr et al. (1985, as cited in [IARC, 1998]), nine cases of acute nonlymphocytic leukemia or preleukemia were observed in 602 patients treated predominantly with CP for non-Hodgkin's lymphoma as compared with the expected incidence of 0.12 cases in the general Danish population. Similarly, Hass et al. [1987] reported that the relative risk of leukemia in women with ovarian and breast cancer was 1.5, 3.3, and 7.3 after receiving cumulative doses of 10, 10–29, or >30 g CP, respectively [IARC, 1998].

Findings from carcinogenicity studies conducted in rodents and epidemiological biomonitoring in humans indicate that there is sufficient evidence for the carcinogenicity of cyclophosphamide in rodents and humans.

APPLICATION OF THE CANCER GUIDELINES MOA FRAMEWORK

Steps in the Cancer Guidelines framework for determining whether a postulated MOA is operative include an outline of the sequence of events leading to cancer, identification of the key events, and determination of whether there is a causal relationship between events and cancer (e.g., dose-response relationship and temporal associations). Additionally, the plausibility of the hypothesis and the examination of other potential MOAs are explored. Finally, the relevance, if any, to humans is assessed.

Description of the Postulated Mode of Action

Based on the available information, it is postulated that CP causes a wide range of neoplasms at various sites including tumors of the bladder, and the hematopoietic and nervous systems of rats and tumors in the mouse lung (male and female) and hematopoietic system (males) through the induction of a mutagenic response. A similar hypothesis can be made for bladder cancer and possibly other cancers observed in humans treated with CP. These observations coupled with the wealth of consistently positive in vitro and in vivo genetic toxicity data allow the formulation of the above hypotheses and the development of the MOA analysis. Because of the wealth of human data, the approach departs from the standard framework analysis and allows a discussion of animal findings followed by human data on key events and other relevant steps in the framework analysis. In this manner, the surrogate rodent data can be used as support when there are gaps in the analysis of human data and the issue of human relevance can be addressed as the framework analysis progresses. It is further assumed that a single MOA is operative regardless of the site of tumor induction. Several pieces of evidence defend this approach: (1) there is a wealth of positive human data on mutagenicity and carcinogenicity of CP; (2) the primary tumor of interest (bladder cancer) induced by CP occurs in rats and humans; (3) key events linked to a mutagenic MOA for CP have been reported in the published literature (e.g., in vivo gene mutations and chromosomal aberrations in rodents and humans); and (4) the metabolism and distribution of CP is similar in humans and rats [Anderson et al., 1995].

Key Events

The postulated key precursor events linked to tumor induction by CP involve the following: the metabolic formation of the main alkylating species, PAM (see Fig. 2), DNA damage (via alkylation) after metabolism of CP by rodents and humans, followed by induction of multiple adverse genetic events (gene mutation and chromosomal aberrations) associated with cytotoxicity, which leads to regenerative proliferation (i.e., increased cellular hyperplasia, cell proliferation, and increased organ weights), and ultimately progresses to urinary bladder tumors (Fig. 4). Each of these key events is discussed in the next section of this article.

Metabolism to Phosphoramide Mustard, PAM, and/or Acrolein

As stated earlier in "genotoxicity data collection" section and depicted in Figure 2, CP is oxidized by P-450 mixed function oxidases to 4-hydroxy-CP (4-OHCP), further oxidized to carboxyphosphamide, which gives rise to a potent alkylating agent at a low pH, nornitrogen mustard. Another pathway described by Anderson et al. [1995] is the oxidation of 4-OHCP to aldophosphamide, which undergoes β-elimination to yield PAM and acrolein. PAM forms a reactive aziridium ion and is listed by Anderson et al. [1995] as the metabolite responsible for the antineoplastic response and alkylation of DNA [Anderson et al., 1995]. Acrolein, which is produced in equimolar amounts to PAM, is listed by Cox [1979] as highly toxic and thought to be involved in the toxic side effects of CP. Several observations, however, suggest that acrolein might be a coparticipant in the carcinogenicity of CP [Cohen et al., 1992] suggested that acrolein might have weak, promoting and complete carcinogenic activity. By contrast, as stated in the World Health Organization [WHO, 1991] Health Criteria for acrolein, "the reactivity of acrolein confines it to the site of exposure and pathological findings are limited to these sites." This reflects the findings from long-term studies showing no tumors when acrolein was administered via the oral route to rats or dogs [Toxnet SIS, 2003]. Furthermore, while acrolein is mutagenic in vitro, results from in vivo genetic toxicology inhalation studies were negative in nasal mucosa, the site of exposure in this study [Toxnet SIS, 2003]. This metabolite is, therefore, ruled out as the putative mutagen. It may, however, contribute to the carcinogenic properties of CP through cytotoxicity as suggested by the findings of Cox [1979] showing that acrolein is the causative agent of CP-induced cystitis in the rat. In this study, Wistar rats of both sexes were dosed i.p. with the CP analog, diethyl CP, which generates acrolein but not PAM. Forty-eight hours after dosing, rats had significant bladder damage (as measured by dry or wet bladder weight) compared with the controls. The author speculated that in CP-treated rats, acrolein is produced in situ in the urothelial cells during breakdown of aldophosphamide (Fig. 2), a CP metabolite that produces cytotoxicity.

Distribution of CP in the body and its metabolism are similar in humans and various animal species including rats [Brock et al., 1971] as cited in [Anderson et al., 1995]. However, individual variation in metabolism has been reported in adults and pediatric patients who appear

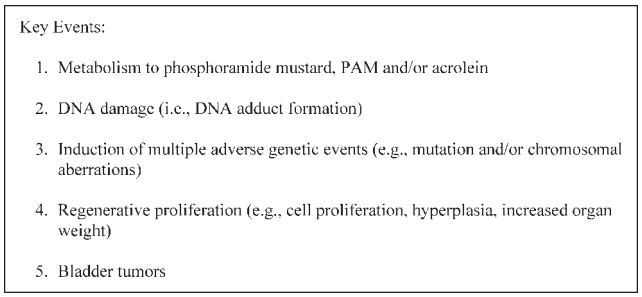

Fig. 4. Proposed key events in the mutagenic mode of action analysis for cyclophosphamide.

to metabolize CP faster than adults [Tasso et al., 1992] as cited in [Anderson et al., 1995].

DNA Damage

A biologically relevant level of DNA adduction must be achieved in the appropriate target cells within the initial few hours of exposure to initiate cancer [Ashby et al., 1993]. However, the presence of DNA adducts alone does not establish a link to tumorigenesis but it is a key step in the CP cancer process and is clearly demonstrated in animals.

Animals. CP does not bind to DNA in the absence of S9 activation [Benson et al., 1988]. This finding is in agreement with the data from the in vitro genetic toxicology assays and suggests that only the metabolite(s) are DNA reactive. As an alkylating agent in vivo, CP cross-links strands of DNA, particularly at the N-7 position of guanine. Povirk and Shuker [1994] in their review of mutagenesis induced by nitrogen mustards state that the major alkylating product of CP appears to be PAM. PAM, in turn, gives rise to a series of mono and cross-linked adducts that rapidly lose the phosphate group and resemble the nornitrogen mustard adducts. As stated earlier by Benson et al. [1988], the PAM metabolite of CP interacts directly with guanine, producing a DNA adduct (hydroxylated nornitrogen mustard adduct) in rat bladder between 2 and 7 hr after treatment with 2.775 MBq [^{3}H] CP. The highest concentration of DNA adducts (three to eight times higher than adducts formed in the kidney) was found in the lungs of mice treated with 100 μCi CP 2–7 hr after i.p. injection [Hemminki, 1985]. Povirk and Shuker also state that no single DNA lesion has been implicated as the primary lesion responsible for nitrogen mustard-induced mutagenesis although adenine and guanine adducts seem to be involved. Furthermore, Maccubbin et al., 1991, found that CP forms phosphotriester adducts at a relatively high frequency (67% of total DNA alkylation); the biological significance of this formation is unknown. Nevertheless, DNA adduct formation at the primary target site has been demonstrated in both rodent species within the initial few hours of exposure. Anderson et al. [1995] in their review of CP mutagenicity data state that at the DNA adduct level, the genotoxic activity of CP results from metabolic activation to the highly reactive metabolite, PAM. This metabolite binds to DNA forming labile covalent DNA adducts and intra-/interstrand cross-links. Interstrand cross-links, in particular, have been shown to result in blockage of DNA replication and are widely considered to be responsible for the cytotoxic action of this compound.

Temporal and site concordance for bladder tumors has been demonstrated in the mouse comet assay performed by Tsuda et al. [2000]. In this study, groups of four male ddY mice received a single i.p. injection of 100 mg/kg CP and were sacrificed 3, 8, or 24 hr postexposure. Significantly increased DNA damage in urinary bladder tissue, as measured by the mean migration of DNA fragments in this single cell gel electrophoresis assay, was recorded 8 and 24 hr posttreatment.

Humans. No data were found in the open literature on DNA adduct formation in humans; nevertheless, SCE in lymphocytes from cancer patients [Schuler et al., 1979; Musilova et al., 1979; Duker, 1981] have been reported by several investigators (see Table II). By virtue of the alkylating activity of CP and the data presented earlier showing that mutagenesis is induced in rats, mice and humans, it is reasonable to assume that DNA adducts and other forms of DNA damage occur in humans and lead to fixed mutations.

Mutation/Chromosomal Aberrations

Animals. As discussed in "genetic toxicology" section, there is a vast array of data showing that CP is mutagenic and clastogenic both in vitro and in vivo. In fact, CP is frequently used as a positive control for both in vitro and in vivo rodent cytogenetic assays and induces significant and/or dose-related increases in SCE, chromosome aberrations, and/or micronuclei primarily in bone marrow harvested from mice, rats, or hamsters following single or multiple exposures. In the in vivo transgenic gene mutation assay, CP was selected by Myhr [1991] to demonstrate induced mutations in one of the first transgenic models developed because of its mutagenic/carcinogenic properties and other investigators have used CP to demonstrate tissue specificity of mutagenesis. Although no transgenic rat models assaying CP are available, this does not compromise the effort because the observed mutagenic response in mice is consistent with the positive effects detected in multiple animal species (mice, rats and hamsters) and CP was clearly identified as a systemic mutagen.

Humans. In humans, chromosome damage has been seen in peripheral blood lymphocytes (PBL) and/or bone marrow from patients and/or workers exposed to CP. Anderson et al. [1995] state that the lowest effective dose was 35 mg/kg for human lymphocytes (as reported by [Bochkov et al., 1986] and 40 mg/kg for bone marrow cells recovered from five patients suffering from various malignancies (as reported by [Goetz et al., 1975]. Anderson et al. [1995] also cite the findings of Hettner [1992] that indicated an increase in *Hprt* gene mutations (~4X higher than matched controls) in the PBL of 23 workers occupationally exposed to CP. Likewise, mutations of the tumor suppressor *p53* gene have been reported in nine of 10 patients with CP-associated bladder tumors [Kahn et al., 1998]. There is also evidence of accumulation of p53 protein in L929 mouse fibroblasts treated with 10 μg/ml CP [Frische et al., 1993]. Hellmich et al. [2005] speculate that alterations in the tumor suppressor *p53* gene are

induced primarily by chromosomal aberrations or mutations and are likely mechanisms associated with earlier events in tumor induction. While such mechanisms are common in most malignancies, it remains unproven whether mutations of the *p53* gene are directly related to CP exposure. Nevertheless, the presence of a mutated *p53* gene plays an important role in the development and possible progression of human bladder cancer [Jung and Messing, 2000]. Thus, the presented data support the proposal that CP induces mutations in humans and animals via a mutagenic MOA; once the mutated cells replicate, a complex series of events associated with tumor production ensues. These events include: cytotoxicity, regenerative proliferation, and tumor formation.

Cytotoxicity

There is sufficient evidence to demonstrate that CP metabolites are cytotoxic at higher doses; however, cytotoxicity does not appear to be an obligatory step in the process of tumor progression since CP-induced mutagenicity is observed in the absence of cytotoxicity.

Animals. CP and its major metabolites (PAM and/or acrolein) are well-known cytotoxicants and in vitro mutagens (see "in vitro test systems" section). In toxicity testing, Phillips et al. [1961] administered 222 mg/kg CP via a single i.p. dose to 28 male CFN rats; animals were observed for 13 days postinjection. Ten of the 28 mice died within 11 days and the remaining animals were killed at selected intervals over the 13-day observation period. Results show that within 1 day of treatment, urinary bladder damage, consisting of numerous hemorrhages and significantly increased wet organ weight, was apparent. Microscopic evaluation of the day 1 bladders revealed extensive ulceration of the mucosa. The investigators also reported that ulceration remained prominent through the 6th day but repair and regeneration "were already evident at 4 days, when mitotic activity was seen in the epithelium outside of zones of ulceration." The dose response to the pathogenesis of bladder damage was also studied in groups of three to six male CFN rats receiving single i.p. injections of 29, 44, 66, 100, 148, or 222 mg/kg CP. These data show that doses as low as 29 mg/kg ($\sim$0.16 of the LD_{50}) were toxic to the bladder; all six rats in this group had bladder hemorrhages and significantly increased wet organ weight. Phillips et al. [1961] also conducted studies with a group of 17 male and female dogs injected i.v. with CP as follows: 2 at 100 mg/kg; 13 at 50 mg/kg; 2 at 25 mg/kg. Two days after treatment, 16/17 dogs had bladder damage; bladders were reported to be thick and edematous with large areas of hemorrhage scattered throughout; three bladders had focal regions of necrosis.

Humans. Humans largely excrete CP via the kidney; however, the metabolic products can accumulate during kidney failure. Bladder toxicity is well known in humans treated with CP causing hemorrhagic cystitis in the bladder of rheumatoid arthritis and lupus erythematous patients (Bennett, 1974 and Plotz et al., 1979 as cited in IARC [1981]) and in children treated for neoplasia (Johnson and Meadows, 1971 as cited in IARC [1981]). It is thought to be associated with contact of the urothelium with the highly toxic, acrolein metabolite and may represent a precancerous state (Cox, 1979 as cited in Anderson et al. [1995]). In agreement with Cox [1979], IARC also states that the bladder toxicity of CP is due to the formation of acrolein. Another possible side effect is the development of a secondary cancer, typically of the bladder, lymph nodes, or bone marrow. Secondary cancers may occur up to several years after drug treatment has ceased [McEvoy, 2002]. It is toxic to the unborn fetus and should not be used during pregnancy because CP is genotoxic to the germ cells of mice, rats and hamsters [Anderson et al., 1995]. CP also interferes with normal cell function and kills rapidly dividing cells such as developing bone marrow cells and stimulated lymphocytes engaged in proliferation [Kitchell, 2005]. Nevertheless, because of its marked ability to kill rapidly dividing cells, it has been used with success in cancer chemotherapy.

Regenerative Proliferation

Regardless, destroyed cells must be replaced and it is likely that a major loss of normal cells through cytotoxicity associated with mutagenicity would provide a selective advantage for the viable mutated cells. The replacement of lost cells initiates the fourth key event, regenerative cellular proliferation. Possible activities that signal this key event and can be measured include: hyperplasia, increased mitotic activity, and cell proliferation leading to increased organ weight.

IARC cites the data from several studies [Phillips et al., 1961; Koss, 1967; Campobasso and Berrino, 1972] indicating that administration of a single i.p. dose of CP caused marked necrosis of the urinary bladder in rats and dogs. In CFN male rats, Phillips et al. [1961] described the mucosa of bladders, 1 day posttreatment with a single i.p. dose of 222 mg/kg, as containing numerous hemorrhages; bladders were "thickened and spongy, and their wet weight was significantly increased." By day 4, repair and regeneration was evident as indicated by increased mitotic activity. Even after day 8, increased mitotic activity was persistent.

Following the work of Phillips et al., Koss [1967] administered a single i.p. injection of 200 or 400 mg/kg CP to Swiss white male and female rats. These investigators noted "a constant and reproducible sequence of events in the urinary bladder," consisting of necrosis of the epithelium (1 hr after treatment) followed by an "extremely rapid" epithelial regeneration and hyperplasia. By 36 hr, regeneration was "well in evidence" and by

48 hr, hyperplasia was well established. By the end of the study (25 days), Koss stated that "... the epithelium showed a markedly atypical papillary hyperplasia." Similar evidence of cytotoxicity leading to a proliferative response in mouse bladder was reported by Campobasso and Berrino [1972] within 3 days of a single i.p. administration of 10 mg/kg CP to male and female Swiss mice. At the end of treatment (10 injections of 1.25 mg/kg CP over a 2-week period; total dose equals 12.5 mg/kg), high mitotic activity was evident 15 days after the one-dose exposure and 10 days after the 10-dose exposure.

Bladder Tumors

As noted earlier, significant increases in malignant tumors, which were primarily transitional cell carcinomas of the urinary bladder in male rats, were seen at doses $\geq$1.25 mg/kg/day CP [Schmähl and Habs, 1979]. Slight but not significant increases were also observed at the lower levels ($\leq$0.63 mg/kg/day) (Table III).

Dose-Response Relationship

Mutagenic Events

As noted in "gene mutation assays" section, the lowest effective dose for induction of SCEs in rat bone marrow is a single administration of 0.62 mg/kg [Simula and Priestly, 1992]. These data are consistent with the data of Schmähl and Habs [1979] demonstrating tumor formation (primarily transitional cell carcinoma of the urinary bladder) at levels of 1.25 and 2.5 mg/kg in Sprague Dawley rats. The total doses for this lifetime study were 475, 698, or 1,270 mg/kg.

In humans, Bochkov et al. [1986] reported chromosome aberrations and SCEs in lung cancer patients 2 hr postexposure to the initial treatment with single i.v. doses of 33–40 mg/kg CP, and Kahn et al. [1998] found mutations in the *p53* tumor suppressor gene in resected or biopsy tissue from the urinary bladders of patients receiving cumulative doses as low as 6 g. These data are in harmony with the results of Travis et al. [1995] who studied the relationship between dose and effect within a cohort of 6,171 survivors of non-Hodgkin's lymphoma treated with CP. Of these individuals, 48 developed cancer of the urinary tract. On the basis of their analysis, the investigators concluded that among patients who had received a total amount of CP $<$20 g, 20–49 g, or 50 g, there was a 2.4-, 6-, or 14.5-fold risk of bladder cancer, respectively.

Regenerative Proliferation

Cytotoxicity leading to a proliferative response, manifested as "extremely rapid" epithelial regeneration and hyperplasia, was seen by Koss [1967] in the urinary bladder of male and female rats within 48 hr of receiving single doses of 200 or 400 mg/kg CP. A similar argument can be made for this key event (i.e., proliferation occurs at CP doses lower than the total lifetime doses causing bladder tumors).

Temporal Associations

Throughout this discussion, the rapid appearance of key events is striking. For example, a time course study, conducted by Dearfield et al. [1985] in the bone marrow of Fischer 344 rats dosed with 5, 10, or 20 mg/kg CP, showed a gradual time-dependent increase in SCEs starting as early as 30 min after exposure. In humans, Bochkov et al. [1986] demonstrated chromosome aberrations and SCE induction in lung cancer patients within 2 hr of CP treatment. Similarly, regenerative proliferation occurs early [e.g., bladder damage within 1 day and repair and regeneration of the rat bladder within 4 days of treatment with a single i.p. dose of 222 mg/kg CP [Phillips et al., 1961]]. Finally, malignant tumors were seen in male and female rats after 60 or 40 weeks of treatment, respectively [Schmähl and Habs, 1979]. These studies have been selected as representative and overall indicate that within 1 day of exposure, a cascade of events is set in motion that leads to mutagenesis, cell proliferation and tumors.

Plausibility and Coherence of the Database

With the currently available information and the acknowledgment that some pieces of the database are missing (DNA adduct formation and regenerative proliferation in humans), a mutagenic MOA for urinary cancer in rats and humans remains plausible and coherent. As shown in Figure 3, CP is positive in virtually every in vitro or in vivo genetic toxicology assay in a wide variety of species. There are data for key events leading to tumors and all of these key events have been demonstrated qualitatively and quantitatively in animals and are plausible in humans. While a recovery experiment* was not found in the open literature, there is evidence suggesting that CP-associated cancers may occur in humans up to several years after drug treatment has ceased [McEvoy, 2002]. Finally, there is concordance between the tumor profile for mutagenic carcinogens and CP. Typically, mutagenic carcinogens induce tumors in multiple sites, multiple species and both sexes [Tennant, 1993], and the time to tumor induction is generally reduced as compared with nonmutagenic carcinogens. These characteristics fit the tumor profile for CP.

*Typically, a recovery experiment includes a satellite group of animals treated for the duration of the study then held for a defined period of time without treatment. As stated in the 2005 EPA Cancer Guidelines, the function of this group is to assess whether "in the absence or reduction of carcinogenicity, the event is blocked or diminished." Accordingly, these tests are considered useful for the association between events and the tumor response.

Alternative Modes of Action

Several MOAs have been established for urinary bladder carcinogenesis in rodents; these include DNA reactivity (the proposed MOA), oxidative stress, cytotoxicity, and urinary calculi. In case of CP, no available data exists to support oxidative stress as a possible mode of action.

With respect to cytotoxicity, CP metabolites such as PAM and acrolein are highly cytotoxic. Therefore, cytotoxicity, which is a step in oxidative stress, is listed as an associative event in our proposed MOA. As Butterworth [2006] states, "When a mutagen is given at doses that are cytotoxic, DNA reactivity in combination with increased cell turnover, work synergistically to dramatically increase the mutagenic and carcinogenic potential." While it is likely that cytotoxicity may provide a selective advantage for the mutated cells, CP is, nevertheless, mutagenic at noncytotoxic concentrations in vitro and in vivo.

Bladder tumors arise secondary to urinary calculi - no data are available to support this MOA for CP. Similarly, most of the agents that induce tumors through this MOA are not mutagenic (e.g., uric acid, calcium oxalate, uracil, thymidine, and melamine).

The above analysis indicates that there is little evidence that would justify a further consideration of these alternative MOAs. Accordingly, no other MOA is postulated for CP.

Relevance to Humans

CP was designated as carcinogenic by IARC in 1981 and there is convincing evidence that CP operates through a mutagenic MOA in both humans and rodents. IARC [1981] further states that the organ-specific effects of CP on the bladder are the same in animals and humans, and carcinogenic doses in animals are lower on a milligram per kilogram basis than therapeutic doses in some human studies. For example, human therapeutic doses range from 1–5 mg/kg/day (oral) to 10–15 mg/kg 3X/week (i.v.). Doses as high as 144–270 mg/kg have been given more than 4 days to condition patients for bone marrow transplants [IARC, 1981]. Accordingly, a linear extrapolation was calculated for CP by Sessnik et al. [1995], based on the data of Schmähl and Habs [1979]. The unit risk (kg body weight/mg CP) is 3×10^{-8}. This represents, on an annual basis, a cancer risk of 1.4 to 10 per million.

Application of the Supplemental Guidance

In addition to the cancer slope factor, the age dependent adjustment factors (ADAF) should be applied because data are available showing a higher frequency of MPCEs in fetal blood (66-fold ↑) as compared with maternal bone marrow (15-fold ↑) following a single administration of 20 mg/kg CP in a mouse transplacental micronucleus assay [King and Wild, 1979] and other findings indicating a threefold increase in chromosome breakage in 26 pediatric noncancerous patients (ages 2–17 years) 4–6 week after treatment with 3–5 mg/kg CP [Dobos et al., 1974].

SUMMARY AND CONCLUSIONS

Evidence of mutagenicity as the MOA for carcinogenesis induced by CP can be summarized as follows:

1. CP is an indirect acting alkylating agent, which requires metabolic activation to the mutagenic species
2. It is a powerful mutagen and/or clastogen at all phylogenetic levels.
3. It induces dose-related increases in DNA adduct formation in the target tissue of exposed rodents.
4. It induces consistent dose-related increases in the frequency of chromosomal aberrations in the peripheral lymphocytes and bone marrow of rodents and humans.
5. Mutagenesis in somatic cells is supported by positive gene mutation and heritable translocations assays in the germ cells of *D. melanogaster* and rodents.
6. It has been associated with malignancies of the urinary bladder in rats and humans, and it induces other tumors at multiple sites, in multiple species of both sexes.
7. The postulated key precursor events connected with tumor induction by CP involve the following: the metabolic formation of the main alkylating species (PAM), DNA damage after metabolism of CP by rodents and humans, followed by the induction of multiple adverse genetic events (gene mutation and chromosomal aberrations), which is often associated with cytotoxicity and progresses to regenerative proliferation (manifested as increased cellular hyperplasia, cell proliferation, increased organ weights). This enhanced DNA replication contributes to the accumulation of multiple genetic errors that ultimately result in urinary bladder tumors. These events have been demonstrated qualitatively and quantitatively in rodents. With the exception of DNA adduct formation and regenerative cell proliferation, these events have also been demonstrated in humans. Thus, the lack of human data for these events does not compromise the framework analysis.
8. There is concordance between doses causing tumors and dose response and temporal association.
9. No convincing data were found for an alternative MOA.
10. Recovery experiments were not found in the open literature but there is evidence suggesting that CP-associated cancers may occur in humans up to several years after drug treatment has ceased.
11. There is concordance between the tumor profile for mutagenic carcinogens and CP (i.e., tumors in multi-

ple sites, multiple species and both sexes and a reduced time to tumor induction).

Therefore, it can be concluded that CP through its metabolites acts via a mutagenic mode of action for carcinogenicity.

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